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Dissociation and unfolding of Pi-class glutathione transferase

Evidence for a monomeric inactive intermediate

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The dissociation and unfolding of the homodimeric glutathione transferase (GST) Pi from human placenta, using different physicochemical denaturants, have been investigated at equilibrium. The protein transitions were followed by monitoring loss of activity, intrinsic fluorescence, tyrosine exposure, far-u.v. c.d. and gel-filtration retention time of the protein. At low denaturant concentration (less than 1 M for guanidinium chloride and less than 4.5 M for urea), a reversible dissociation step leading to inactivation of the enzyme was observed. At higher denaturant concentrations the monomer unfolds completely. The same unfolding behaviour was also observed with high hydrostatic pressure as denaturant. Our results indicate that the denaturation of GST Pi is a multistep process, i.e dissociation of the active dimer into structured inactive monomers followed by unfolding.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) are a family of dimeric multifunctional proteins that facilitate the nucleophilic attack of GSH by a large variety of reactive electrophiles (Chasseaud, 1979; Jakoby & Habig, 1980; Mannervik, 1985). On the basis of several physicochemical and immunological criteria, the considerable number of cytosolic mammalian GSTs so far characterized can be grouped into at least four distinct classes: Alpha, Mu, Pi and Theta (Mannervik *et al.*, 1985; Meyer *et al.*, 1991). Crystals of Pi-class GST have been obtained for the enzymes purified from human placenta (GST Pi) (Parker *et al.*, 1990), bovine placenta (Schaffer *et al.*, 1988) and pig lung (Reinemer *et al.*, 1991). From three-dimensional structural analysis, at 0.3 nm (3 Å) resolution, carried out with crystals obtained from the pig lung enzyme, it was found that each subunit (207 residues) is folded into two domains of different structure (Reinemer *et al.*, 1991). Kinetic (Mannervik & Danielson, 1988) as well as structural (Reinemer *et al.*, 1991) investigations demonstrated that each individual subunit contains a complete active site and displays kinetic properties that are independent of its neighbouring subunit in the native dimer. Nevertheless, at present, there is no information about the properties of individual subunits in solution.

The major purpose of the present study was to determine the interrelationship between the dissociation and unfolding steps of GST Pi by using different chemical and physical denaturants. We obtained evidence consistent with the presence of a structured inactive monomer at equilibrium, indicating that the dimeric arrangement of the enzyme is of fundamental importance for its catalytic capacity.

EXPERIMENTAL

Protein and reagents

GST Pi was purified from human placenta by sequential affinity chromatography, f.p.l.c. and gel filtration (Caccuri *et al.*, 1990). Protein concentrations were determined by the method of Bradford (1976). Guanidinium chloride (GuCl) and urea were obtained from Sigma Chemical Co. Urea was recrystallized before preparation of stock solution. All solutions were filtered before the experiments by using a 0.2 μm porosity inert filter.

Enzyme activity

Different amounts of GST Pi (0.06–3 μM) were incubated for at least 30 min at 25 °C with 0–8 M-urea or 0–4 M-GuCl in 0.1 M-potassium phosphate buffer (pH 7.0)/1 mM-EDTA/1 mM-dithiothreitol (buffer A). Enzyme activity was measured by the methods of Habig & Jakoby (1981) with 2 mM-GSH and 1 mM-1-chloro-2,4-dinitrobenzene. Since with increasing urea or GuCl concentrations the non-enzymic rate decreases, each activity was corrected for the corresponding blank. To study renaturation, a small amount of denatured enzyme was rapidly diluted (1:100) into buffer A and the activity measured after 10 min.

Intrinsic fluorescence

The intrinsic fluorescence of the protein was measured with a Jobin Yvon 3D fluorimeter equipped with a thermostatically controlled sample holder. GST Pi (3 μM) was incubated for 30 min at 25 °C in buffer A in the presence of different concentrations of denaturants before spectral measurements. Spectra were recorded in 1 nm wavelength increments, and the signal was acquired for 1 s at each wavelength. For each sample, the spectrum was corrected by subtraction of the spectrum for buffer A alone. The samples were excited at 280 ± 3 nm. The absorbance at 280 nm for the highest concentrations of GST Pi used in the experiments was only 0.15, and a change smaller than 5% in absorbance accompanied the unfolding. Thus no corrections in the spectra were made for inner filter effects. To study refolding, denatured protein was diluted (1:2) into buffer A and fluorescence spectra were recorded after 10 min.

Tyrosine exposure

The fractional tyrosine exposure was estimated as described by Ragone *et al.* (1984). Second-derivative absorption spectra were recorded at 0.2 mg of protein/ml on a Jasco 7800 model spectrophotometer.

Abbreviations used: GST, glutathione transferase; GuCl, guanidinium chloride.

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High pressure

The effect of pressure on enzyme activity was determined with the pressure bomb previously described (Hui Bon Hoa *et al.*, 1982). These experiments were performed in 50 mM-Tris/HCl, pH 8 (buffer B). This buffer, unlike acetate or phosphate buffers, maintains a H^+ concentration that is almost pressure-independent. The residual activity of pressurized enzyme (8 min of incubation) was measured at ambient pressure after rapid decompression (20 s) and cell bomb opening (90 s).

Gel filtration

The gel-filtration experiments were performed at 25 °C using a Protein Pak 300 sw (Waters) column (0.78 cm × 30 cm). The following proteins of known molecular mass were used as standards: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), BSA (67 kDa). Chromatographic measurements were made by using an h.p.l.c. system equipped with a Rheodyne injector and a photodiode variable-wavelength detector (Kontron). Samples (20 μl containing 3 μM-GST Pi preincubated for 30 min in buffer A supplemented with 0–3 M-GuCl) were injected into the column pre-equilibrated and eluted isocratically with the incubation buffer.

Circular dichroism

C.d. spectra at a protein concentration of 0.28 mg/ml were recorded on a Jasco J 600 model with cuvettes of 0.1 cm pathlength. All measurements were repeated five times.

RESULTS

Unfolding–refolding transition monitored by enzyme activity

The unfolding transition of GST Pi was followed by measuring the loss of enzyme activity in the presence of increasing amounts of GuCl or urea after 30 min of incubation, a time sufficient to achieve equilibrium. The midpoint values were 0.3 M for GuCl and 3.7 M for urea at 3 μM-protein (Fig. 1). The enzyme was completely inactivated at 1 M-GuCl and 4.5 M-urea. At concentrations lower than 1 M for GuCl and 4.5 M for urea, the enzyme inactivation for both chaotropic agents was completely reversible by rapid sample dilution (1:100) (Fig. 1). At higher concentrations of denaturant, the recovery of enzyme activity after rapid dilution was between 75 and 90% for urea and 40 and 55% for GuCl (Fig. 1). It should be noted that the midpoints of the transition curves for both denaturants decreased with decreasing protein concentration, indicating a bimolecular dissociation (Fig. 1, inserts).

Unfolding–refolding transition monitored by intrinsic fluorescence

The equilibrium transitions of GST Pi were also monitored by following the variation in the maximum fluorescence-emission wavelength (Fig. 1). The intrinsic fluorescence for the native state of the protein was characterized by a maximum emission wavelength at 333 nm ± 3 nm (excitation 280 nm) (Fig. 2). This indicates that the two tryptophan residues present in each subunit of GST Pi (Kano *et al.*, 1987) reside in the hydrophobic environments (Lackowicz, 1983). The addition of increasing concentrations of denaturant at equilibrium caused a red shift of the emission spectra (Fig. 2). At 2.5 M-GuCl and 8 M-urea, a $\lambda_{max.}$ of 350 nm occurred, indicating the complete exposure of the tryptophan residues to aqueous solvent (Fig. 1). The midpoint values were 1.5 M for GuCl and 5.5 M for urea. It should be noted that the structural changes observed by fluorescence occurred at denaturant concentrations higher than that which causes enzyme inactivation (Fig. 1). The fluorescence transitions were found to be totally reversible after treatment with GuCl but only partially reversible after treatment with urea (Fig. 1).

Unfolding transition monitored by u.v. spectroscopy

Considering the high number of tyrosine residues (11 for each subunit) present in GST Pi (Kano *et al.*, 1987), their exposure could be a useful spectroscopic probe for following the denaturation transition of the protein. The fractional exposure of tyrosine residues during denaturation with GuCl was determined by second-derivative absorption spectroscopy (Ragone *et al.*, 1984). Two tyrosine residues per subunit were exposed in

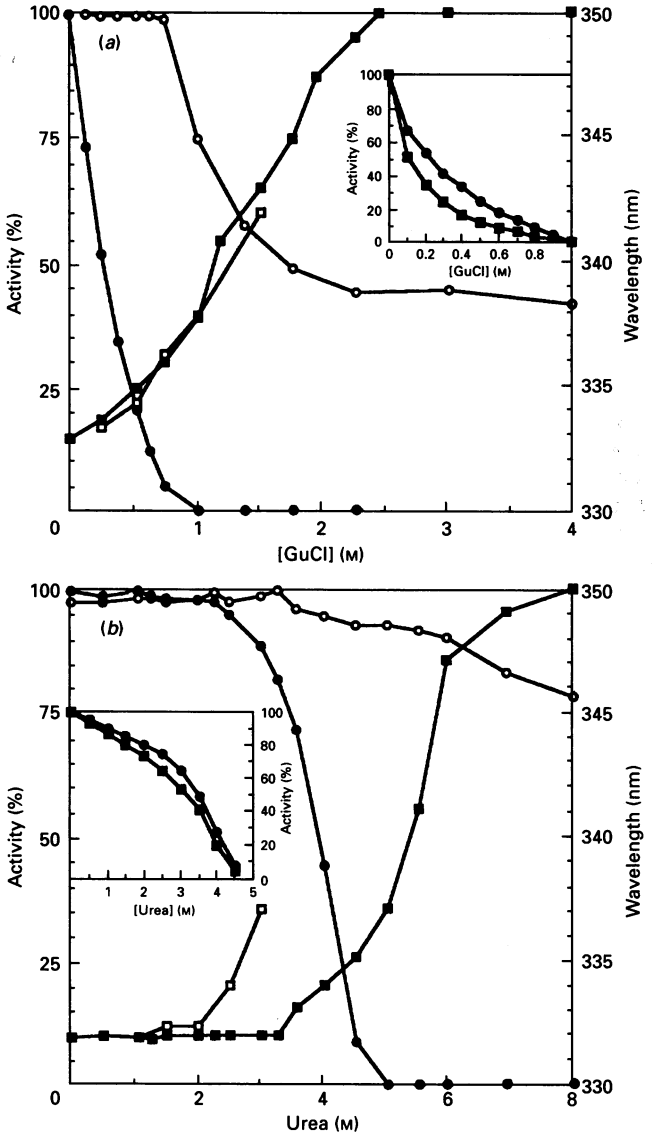

Fig. 1. Activity and fluorescence changes of GST Pi during denaturation treatment

Unfolding was monitored by changes in enzyme activity (●) and $\lambda_{max.}$ displacement of intrinsic fluorescence (■) as described in the text. Solutions were incubated for 30 min at 25 °C in buffer A in the presence of the indicated concentration GuCl (*a*) and urea (*b*). Reactivation (○) of denatured enzyme was followed by rapid dilution (1:100) in buffer A. Enzyme activity was measured 10 min after dilution. The reversibility (□) of $\lambda_{max.}$ displacement was followed after rapid dilution (1:2) of denatured enzyme in buffer A. $\lambda_{max.}$ displacement was monitored 10 min after dilution. Inserts report the inactivation curves at different protein concentrations: 3 μM (●) and 0.07 μM (■) in (*a*) and 3 μM (●) and 0.2 μM (■) in (*b*).

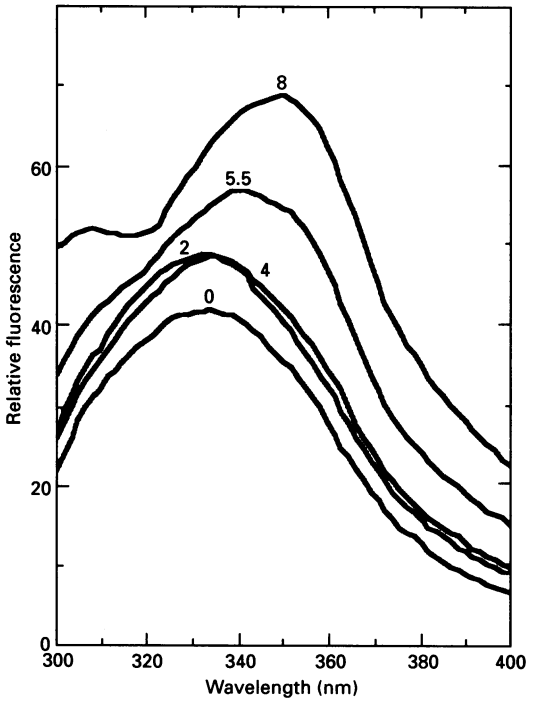

Fig. 2. Fluorescence-emission spectra of GST Pi at different urea concentrations (M)

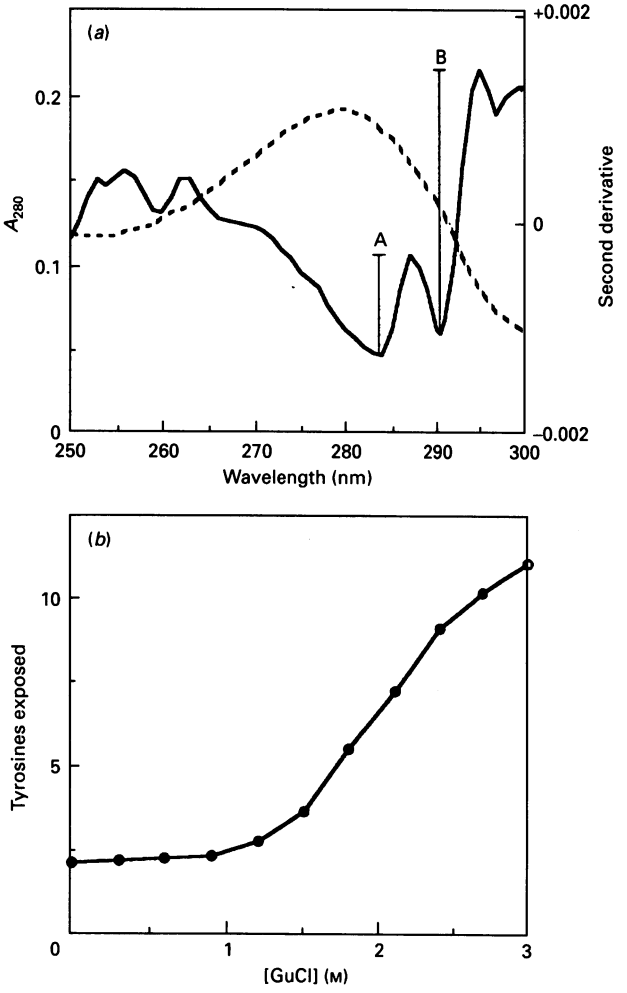

Fig. 3. Second-derivative absorption spectrum of GST Pi

The peak-to-peak distances A to B (*a*) were used to calculate the tyrosine exposure at the indicated GuCl concentration (*b*). ——, Second-derivative absorption spectrum; ----, absorbance spectrum.

the native protein (Fig. 3). At GuCl concentrations higher than 1 M, an increased number of exposed tyrosine residues was found. After the addition of 3 M-GuCl, all tyrosine residues were exposed (Fig. 3). The incomplete overlapping of tryptophan and tyrosine transition curves may indicate that, in the 1–3 M range of GuCl, the following mechanism involves different denatured states of the protein. At low denaturant concentrations, when a bimolecular dissociation has already occurred, no substantial changes in the $\lambda_{max.}$ displacement and tyrosine exposure were visible. This finding excludes the presence of any tyrosine or tryptophan residues at the subunit interface.

Unfolding transition monitored by c.d. spectra.

The effect of the GuCl on the secondary structure of GST Pi was monitored by c.d. spectroscopy in the far-u.v. region. The changes in the far-u.v. c.d. spectra after the addition of GuCl are shown in Fig. 4. No significant changes in the c.d. spectra occurred between 0 and 0.5 M-GuCl. At concentrations of GuCl between 1 and 1.5 M, significant changes in the far-u.v. c.d. spectra were observed, suggesting a substantial loss of the secondary structure of the protein.

Protein unfolding monitored by gel filtration

The quaternary structure of GST Pi was monitored by gel-filtration experiments in the presence of increasing amounts of GuCl (Fig. 5). The apparent molecular mass of the protein decreased from about 45 kDa (elution volume 8.6 ml at 0.0 M-GuCl) to about 29 kDa (elution volume 9.25 ml at 0.9 M-GuCl), indicating that a progressive dissociation of the protein occurs. The absence of a second peak or a shoulder in the elution diagrams (Fig. 5*a*) indicates that the dissociation and unfolding equilibria were rapid compared with the retention times on the column (Endo *et al.*, 1983). Since the molecular mass of the monomer is 23.5 kDa (Mannervik, 1985; Kano *et al.*, 1987), we conclude that the dimer is totally dissociated to partially unfolded monomers at about 1 M-GuCl. When GuCl concentrations were higher than 1.3 M, a gradual decrease in elution volume occurred. Under such conditions, the gel-filtration behaviour reflects the extensive unfolding of the subunits, leading to a decrease in elution volume compared with that of a compact globular structure (Kelly & Price, 1991).

Inactivation of the enzyme by high hydrostatic pressure

In order to verify whether the dissociation step of GST Pi is accompanied by the loss of enzyme activity irrespective of the chemical denaturants used, the inactivation of the enzyme was studied by using high hydrostatic pressure. It is well known that the exposure of oligomeric proteins to high hydrostatic pressure may produce dissociation into monomeric components (Jaenicke, 1987). In Fig. 6 is reported the residual activity of the pressurized GST Pi at various protein concentrations after 8 min of incubation. At protein concentrations higher than 10^{-7} M, no loss in catalytic activity was recorded up to 3.6×10^8 Pa. At lower enzyme concentrations, a decrease in enzyme activity was observed with increasing hydrostatic pressure. Moreover, the midpoint of the transition decreased dramatically with decreasing protein concentration, indicating again a bimolecular dissociation. It should be noted that protein denaturation is normally observed at higher pressure and longer incubation times than those used in our experiment (Murakami, 1970).

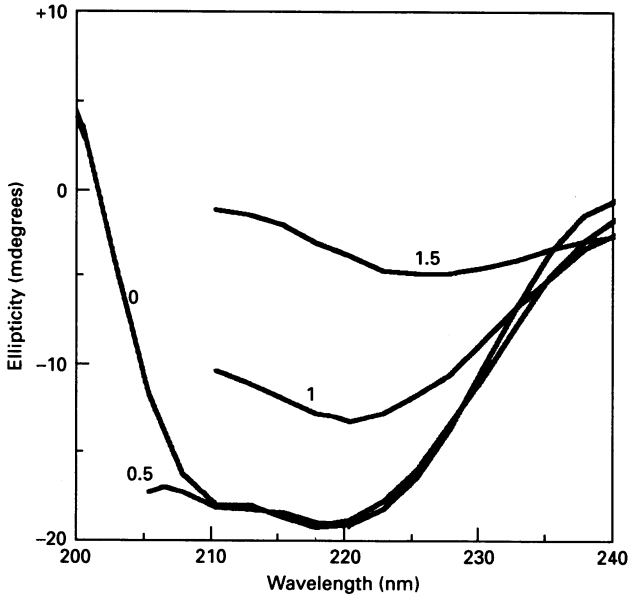

Fig. 4. Far-u.v. c.d. spectra of GST Pi

The spectra were recorded at 25 °C after 30 min of incubation in buffer A in the presence of the indicated concentration (M) of GuCl.

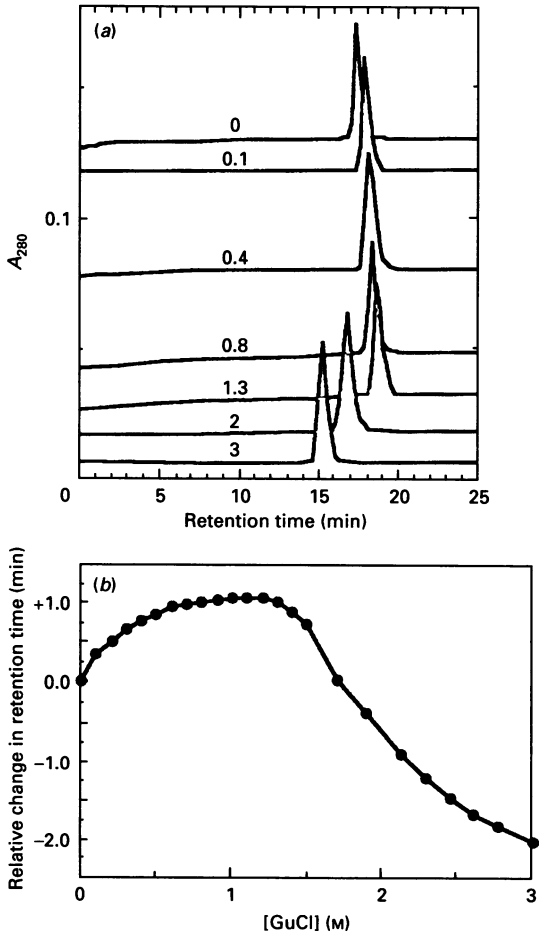

Fig. 5. Dissociation and unfolding of GST Pi as monitored by gel filtration

(*a*) Elution profile of GST Pi at the indicated GuCl concentration (M). (*b*) Changes in the retention time as a function of GuCl concentration.

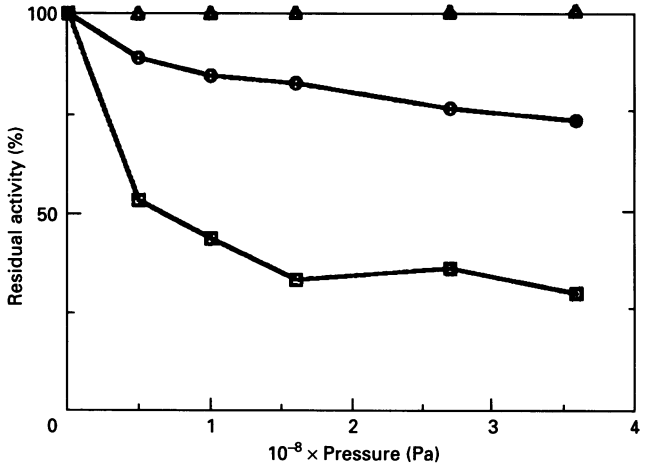

Fig. 6. Activity change in GST Pi activity at different concentrations as a function of hydrostatic pressure

The activity was recorded after 8 min of incubation at 25 °C in buffer B. Protein concentrations were: 10^{-7} M (△); 10^{-8} M (○); 10^{-9} M (□).

DISCUSSION

GST Pi is a homodimeric enzyme in which each individual subunit contains a complete active site and exhibits kinetic properties that are independent of its neighbouring subunit in the native dimer (Mannervik, 1985). In addition, there is evidence that the subunits are structurally independent. In fact, Maruyama *et al.* (1984) showed that the heterodimeric YaYc GST exhibits a c.d. aromatic spectrum that is virtually a composite of c.d. spectra of homodimers YaYa GST and YcYc GST. However, attempts to isolate structured monomeric GST that retains catalytic activity have not been made. The non-coincidence of denaturation curves at equilibrium, as measured by different physical techniques, is one of the methods used to detect the presence of protein intermediates (Pace, 1986).

Our results indicate that denaturation of GST Pi is a multistep process, i.e. dissociation of active dimer into inactive structured monomer followed by unfolding. In fact, at lower concentrations of denaturant, the inactivation is a bimolecular reaction, indicating that the dissociation leads to inactive monomers. This conclusion is further supported by the results of gel-filtration experiments. That the inactivation is due to dissociation of the dimer rather than to an unspecific effect of the chemical denaturants on the active site of the enzyme was confirmed by the hydrostatic pressure experiments. In fact, exposure of GST Pi to hydrostatic pressure again produces evidence for a bimolecular process leading to inactive folded monomers. Therefore, regardless of the chemical or physical denaturants used, the dissociation of GST Pi leads to inactivation but does not implicate any gross structural changes for the monomer, as indicated by the physicochemical methods used. It should be noted that the minor subtle structural changes that occur to the protein on dissociation are completely reversible, without any hysteresis effect, as demonstrated by the complete recovery of the activity by dilution. Thus it may be concluded that the dimeric structure of GST Pi is of fundamental importance for its catalytic function.

During the revision of the present paper, the equilibrium unfolding of pig lung GST was published (Dirr & Reinemer, 1991). In contrast with our results, Dirr & Reinemer (1991) obtained evidence that the dissociation and unfolding mechanism of the pig enzyme are closely coupled, resembling a concerted two-states process with only the native dimer and unfolded monomers. One of the possible explanations for this discrepancy

may be the different experimental assay conditions used. Dirr & Reinemer (1991) added trypsin instead of urea or GuCl to the assay mixture to prevent re-activation of unfolding enzyme. However, the use of trypsin, acting on the unfolded enzyme but not on the native enzyme (Dirr & Reinemer, 1991), does not exclude the possibility that structured inactive monomers refold during the assay measurement. On the other hand, it is generally thought that isolated monomers with small intersubunit interfaces are very stable and therefore undergo minor structural changes on dissociation. In this regard, it has been demonstrated that Pi-class GST has a moderate intersubunit area interface (13 %) in the native form (Reinemer *et al.*, 1991). With respect to the unfolding mechanism, GST Pi behaves similarly to several enzymes, e.g. lactate dehydrogenases, glutamate dehydrogenase and pig heart fumarase (Ma & Tsou, 1991; West & Price, 1988; Kelly & Price, 1991). In these cases, the loss of activity observed on dissociation at moderate denaturant concentration has been attributed to subtle changes at the active site, without any great loss of secondary or tertiary structure, as detected by c.d. and fluorescence. It has been proposed that the active sites of these enzymes are located in limited and relatively fragile regions the conformational integrity of which is more sensitive to denaturation than the molecule as a whole (Tsou, 1986). Active-site fragility and consequently flexibility may well be required for the full expression of the catalytic power of these enzymes. It is likely that structural fluctuations, derived from flexibility of functional groups or chain segments, could also be important for the catalytic mechanism of Pi-class GST.

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